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14. ABSTRACT

New contrast-specific imaging modalities such as harmonic imaging (HI) may improve the accuracy of breast ultrasound. Unfortunately, HI suffers from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by combining injection of an ultrasound contrast agent with SHI.
A dual-transducer pulse-echo system was built to perform *in vitro* SHI measurements and experiments were conducted in a perfusion phantom with realistic neovascular flow velocities (around 2 mm/s). Up to 12 dB of subharmonic signal components were measured. *In vivo* animal experiments comparing SHI in canines to perfusion showed good correlation ($r = 0.57$; $p < 0.0001$) with contrast uptake slopes. Six patients with seven benign breast masses were studied. In SHI there was an almost complete suppression of tissue signals allowing the lesion vascularity to stand out. Finally, SHI perfusion estimates obtained in the human breast lesions ranged from 1.67 to 2.46 ml/min/g.

15. SUBJECT TERMS
Breast cancer, ultrasound imaging, ultrasound contrast agent

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4. INTRODUCTION

The goal of any breast imaging modality is to improve the early detection of tumors and to improve the differentiation between benign and malignant lesions. While x-ray mammography is efficacious in diagnosing a high percentage of breast masses, it also produces a high rate of false positives [1]. The percentage of breast biopsies that are actually malignant vary between 10 % and 35 %. Thus, a technique that reliably differentiates between malignant and benign masses would improve the diagnosis of breast cancer and should, therefore, reduce the number of negative biopsies as well as the trauma of the patients. This proposal will attempt to establish such a technique through the novel and innovative use of subharmonic ultrasound contrast imaging.

Ultrasound imaging is currently an auxiliary modality in breast imaging. It is mainly used to differentiate between cystic and solid lesions [2]. Investigations into the possibility of breast cancer diagnosis based on Doppler ultrasound flow detection have produced mixed results, due to overlap between flow measurements in benign and malignant tumors [3-4]. One problem may be the lack of sensitivity in flow detection in small tumor vessels using ultrasound. This hypothesis is supported by reports in the pathology literature describing angiogenic vascular morphology as an independent predictor of metastatic disease [5].

Ultrasound contrast agents produce increases of 15 to 25 dB in the echo intensities of blood flow signals; especially when combined with new contrast-specific imaging modalities such as harmonic imaging [6-7]. However, harmonic imaging has been found to suffer from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. As an alternative we propose using subharmonic imaging (SHI) by transmitting at the double the resonance frequency ($2f_o$) and receiving at the subharmonic (f_o). SHI has the potential to detect slow, small volume blood flow associated with tumor neovascularity, making early detection and identification of tumors very likely. SHI should have much better lateral resolution due to the higher transmitting frequency and should allow tumor perfusion, a measure of angiogenesis, to be estimated via time-dependent subharmonic fractional blood volume estimates. Hence, the current project proposes to increase the ability of breast ultrasound to differentiate between benign and malignant lesions by using SHI.

Quantifiable parameters of tumor angiogenesis will be estimated from the subharmonic signal intensities. A pulse-echo system will be built to perform SHI and tested in vitro as well as in vivo (in animals). The ability of SHI to depict normal vascularity as well as tumor angiogenesis will also be assessed in rabbits. Currently, the NIH and DOD have funded a study at Thomas Jefferson University into the efficacy of ultrasound contrast in the diagnosis of breast disease. We propose to expand on that project by adding SHI in the third year of this proposal. Not only is the potential of SHI in itself innovative, but because of the NIH/DOD funded study it will be possible to compare a number of new and unique approaches to breast cancer diagnosis i.e., SHI, 2D power Doppler with and without contrast as well as harmonic imaging directly to x-ray mammography. Furthermore, this project is extremely cost-effective because the existing grants coves a majority of the personnel costs as well as all major equipment purchases. The amalgamation of the NIH/DOD project with the current proposal also allows for basic research

into the correlation between SHI flow signals and pathologically detected lesion vascularity. This will enable a deeper understanding of the relationship between tumor neovascularity and ultrasound flow measurements

Consequently, this project proposes the development of a novel contrast specific imaging mode called SHI and the derivation of quantitative tumor angiogenesis estimates from SHI data. The fundamental hypothesis is that the neovasculature of malignant lesions can be visualized and quantified with SHI, thus, improving the diagnosis of breast cancer.

5. BODY

The central hypothesis of this project is that the differentiation between benign and malignant breast lesions can be improved by detection and estimation of tumor neovascularity using contrast enhanced SHI. To investigate this hypothesis SHI will be investigated in vitro and then in vivo in rabbits with VX-2 tumors. Finally, approximately 50 women with breast lesions will be recruited in year three and imaged using contrast enhanced SHI. The specific tasks of the project (as presented in the original Statement of Work) can be found in Appendix I.

First an outline of the methods applied will be given followed by a presentation of the results to date. Finally, the conclusions and future directions of the research will be discussed.

5.1 Methods

In vitro experiments

A pulse-echo system was built to perform SHI and measure the FBV as a function of time (Fig. 1). The setup consists of a pair of confocally positioned broadband focused transducers (diameter: 2.54 cm). A pulse/function generator (8111A; Hewlett-Packard Company, Polo Alto, CA, USA) was used to generate 32 cycle busts with a PRF ranging between 20 and 100 Hz. An RF power amplifier (A150; ENI Technology Inc., Rochester, NY, USA) amplified this signal by 55 dB to generate pressure levels from 0.3 to 1.5 MPa. The transmitting transducer used was a 2.54 cm spherical focused, narrow bandwidth, 5 MHz transducer (13-0508S; Harisonic / Staveley Industries Plc, Croydon, UK). The backscattered signals were picked up using a wide band 2.54 cm spherical focused transducer with center frequency of 2.25 MHz (13-0208R; Harisonic / Staveley Industries Plc, Croydon, UK). This substantially improved the spatial resolution of the system, because scattered signals only come from the microbubbles in the small confocal region of the two transducers (1-4 mm^3 for 2 MHz transmission). The sampling frequency was 20 MHz.

The contrast agent used for this part of the study was Optison® (GE Healthcare, Princeton, NJ). Optison is approved for use in echocardiography by the U.S. Food and Drug administration (for improved endocardial border delineation). It consists of a suspension of perfluoropropane-filled albumin microspheres with a concentration of 6.3×10^8 bubbles/ml and the bubbles have mean diameters in the range of 3 to 5 μm. The frequency of insonation used was 4 MHz, as the resonance frequency of Optison is around 2 MHz and this is the frequency range used in the ultrasound scanner employed for our preliminary SHI work [8-9]. This is in keeping with the

concept of minimum threshold for subharmonic generation when the insonation frequency is twice the resonance frequency. This setup was also employed to investigate the subharmonic signal generation from a new contrast agent QFX (Nanfang Hospital, Guangzhou, China), which consists of perfluorocarbon-filled albumin microbubbles [10]. The tubes used to set up the flow system were polyester shrink tubes (Advanced Polymers Inc., Salem, NH, USA) of two different diameters. The smaller tube had internal diameter of 300 μm ± 25 μm, and wall thickness of about 6 μm and the larger one had internal diameter 1 mm ± 25 μm and similar wall thickness. In this report, only results from the smaller tube will be presented, as these are the most interesting for tumor neovascularity evaluations (and results from the larger tube can be found in our publication [11]).

In order to simulate conditions similar to the angiogenic blood flow in tumors, we used a high efficiency, single use dialysis cartridge (F7NR; Fresenius, Bad Homburg, Germany) as a flow phantom (similar to the work by Hindle and Perkins [12]). This cartridge consists of a few thousand very thin tubes encased in a longer plastic tube. The internal diameter of each of these the thin tubes is 200 μm and they have a wall thickness of 6 μm. An acoustic window was cut into the outer tube to prevent any attenuation of the applied acoustic power. The experimental setup for the dialysis cartridge was similar to Figure 1. The transducers were focused on the hollow tubes directly through the acoustic window of the dialysis cartridge. The cartridge and the entire setup were immersed in water. Care was taken to remove all air bubbles trapped in between the tubes; however, complete removal could not be guaranteed.

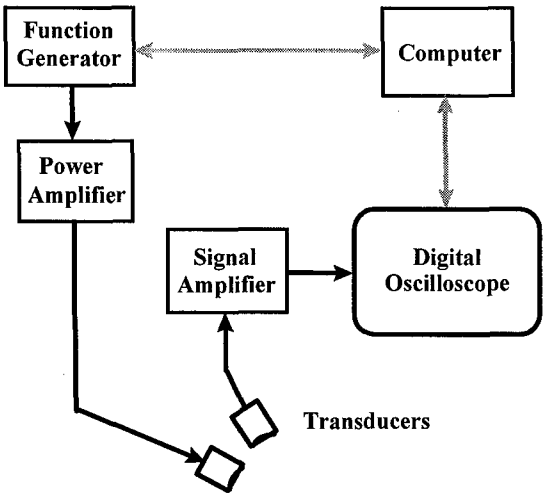

Figure 1. *The dual-transducer pulse-echo system built to perform SHI. A function generator produces a sequence of transmit pulses which are first amplified and then supplied to a single-element broadband focused transducer. Another broadband focused transducer (confocally positioned to the first transducer) will sense signals scattered from the contrast bubbles. The received signals, after being amplified, will be digitized using a digital oscilloscope. The digitized signals are further processed to obtain the subharmonic amplitude (with a bandpass filter) using LabView® (National Instruments, Austin, TX).*

A high pass filter (Krohn-Hite with a cut off at 2.3 MHz) was used at the input side, before the power amplifier to reduce any 2 MHz side bands that may be present. Its effectiveness was confirmed by the absence of any subharmonic component in the backscattered echo from water with varying acoustic pressures. The flow velocity through the hollow tubes of the dialysis cartridge was estimated to 2 mm/s. Backscattered echoes from water and contrast agent were acquired, and the subharmonic components calculated using Matlab.

The contrast agent Optison was also tested in an in vitro flow phantom containing an 8 mm vessel (ATS Laboratories, Bridgeport, CT). In the flow phantom the contrast kinetics of the microbubbles within the flow (i.e., the uptake and washout of the contrast agent) was measured for different concentrations of Optison (1 % and 2 % by volume) and flow rates of 9.8 and 19.6 ml/min and the relative changes were compared.

In vivo animal experiments

Four laboratory bred New Zealand white rabbits (mean weight 3.6 kg) and four laboratory bred mongrel dogs (mean weight 21 kg) were used in this project. The rabbits were sedated with 0.65 mg/kg of a mixture of Xylazine hydrochloride (Gemini, Rugby Laboratory, Rockville Centre, NY) and Ketamine hydrochloride (Ketaset, Aveco, Fort Dodge, IA) administered intramuscularly under the supervision of a veterinary technician. The rabbits were maintained under anesthesia with 15 to 20 mg/kg/hr of 1 % Propofol (Diprivan®, Zeneca Pharmaceuticals, Wilmington, DE) as needed for the entire procedure. The dogs were premedicated with intramuscular administration of a mixture of 0.04 mg/kg atropine sulfate (Anthony Products, Arcadia, CA), and 0.75 mg/kg acepromazine (Promace; Aveco, Fort Dodge, IA). The dogs were placed on a warming blanket to maintain body temperature within normal range. A facemask with Isoflurane 4-5 % (Iso-thesia; Abbott Labs, N.Chicago, IL) was used for induction of anesthesia, which was maintained with 0.5 to 2 % of Isoflurane during the entire procedure. The animal studies were performed under supervision of a veterinarian and fully conformed to the National Institutes of Health guidelines for use of laboratory animals. All protocols were approved by the University's Animal Use and Care Committee.

In vivo experiments were initially carried out in rabbits. However, all 4 animals expired during the placement of the catheters needed for the absolute perfusion measurements (i.e., the gold standard against which SHI was to be compared). We established that the vessels in the rabbits are too small to allow for the required catheter placement. Hence, it was decided to switch the animal model to dogs and a new protocol to that effect was approved by the TJU IACUC. Due to the budgetary restrains of the grant, only 4 animals were evaluated in the perfusion measurements. Moreover, during the imaging phase of the rabbit experiments we found that the SHI software version installed on the Logiq 700 scanner (GE Healthcare, Milwaukee WI) lacked flexibility. Hence, an upgrade was required to run SHI on a more programmable scanner. It was decided to replace the existing Logiq 700 with a completely new Logiq 9 scanner (GE Healthcare, Milwaukee WI) to support this project. This unit was purchased by the Department for clinical use, but it was made available for this research project as well.

The experimental software employed for SHI does not provide any calibrated indication of the acoustic field (such as a mechanical index). Instead, the acoustic output of the 7L transducer was

measured in water at 2 cm's depth (corresponding to the average location of a canine kidney from the skin surface) using a 0.5 mm broadband acoustic hydrophone (Precision Acoustics Ltd, Dorchester, UK). The incident acoustic pressure amplitude was 1.48 MPa (peak negative pressure 0.65 MPa), which corresponds to an *in situ* pressure of approximately 0.9 MPa (assuming attenuation to be 0.5 dB/cm MHz). All imaging parameters were kept constant during injections. For each injection the scan plane was selected to correspond to the maximum diameter of the kidney and a 30 to 40 second digital cine-loop clip, covering baseline to beyond peak enhancement, was acquired. Moreover, sufficient time (10 – 15 minutes) was allowed between injections to avoid any cumulative effect of contrast and to ensure a return to baseline conditions.

Following six contrast injections (3 for each kidney), a neutron activation assay technique based on stable (non-radioactive), isotope-labeled microspheres (initially gold microspheres; BioPhysics Assay Laboratory (BioPal) Inc, Worcester, MA) was employed to quantify the perfusion [13]. The microspheres are 15 μm in diameter and are supplied from BioPal suspended in normal saline containing Tween-80 and 0.01 % Thimerosal. Approximately 10 million microspheres (concentration: 2.5 million spheres/ml) were injected into the left ventricle through the myocardial catheter. This is a small number (fewer than the number of red blood cells in 2 ml of blood) and data reported in the literature show that this procedure does not cause clinically significant embolization [14]. Simultaneously, a reference blood sample was drawn from the catheter positioned in the aorta using an automatic withdrawal pump (Syringe Pump 11 Plus; Harvard Apparatus, Holliston, MA). The withdrawal rate was set at 10 ml/min and collected over a one minute interval resulting in a 10 ml reference blood sample.

Next, a surgically exposed segmental renal artery was ligated (with a surgical suture) to reduce perfusion in the kidney (first on the right and then on the left side). On each side three contrast injections as well as transcutaneous SHI were repeated followed by administration of different isotope labeled microspheres (utilizing Lutetium for the right and Samarium for the left side, respectively). As described above 10 million spheres were injected and a reference blood sample acquired for each side. At the completion of the experiments, the dogs were sacrificed using an intravenous injection of Beuthanasia (0.25 mg/kg).

After euthanasia, the kidneys were harvested, cut in half corresponding to the imaging plane and eight small sections (from the upper pole anterior and posterior, mid-upper pole anterior, ..., lower pole posterior) were extracted. All tissue sections were weighed (the average sample weighed 0.6 g ± 0.17 g) and stored in pre-weighed sample vials. All tissue and blood samples were sent for activation at BioPal's facilities, where they were exposed to a field of neutrons [13]. The samples were stored for 48 hours to allow short-lived activation products to decay. After this decay period, spectroscopic analysis was performed on each sample, and corrections were made to account for interradionuclide crossover and tracer decay during the counting period [13]. The results of the assay were reported as the microsphere concentration of each segment normalized to the microsphere concentration measured in the reference blood sample, which provides a measure of absolute perfusion (Qp; in ml/min g) according to:

$$Q_P = \frac{\frac{\sum spheres\ in tissue}{\sum spheres\ in reference}}{weight\ of\ tissue} \times Q_R \qquad (1)$$

where Q_R is the reference withdrawal rate (in ml/min).

The digital clips obtained from each SHI injection were transferred to a PC for off-line analysis. SHI time intensity curves were acquired in eight regions of interest (ROIs) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) with each ROI corresponding to one of the eight kidney sections (i.e., upper pole anterior, upper pole posterior, mid-upper pole anterior, etc.). To reduce breathing artifacts all time intensity curves were smoothed with a fifth-order moving average filter using Matlab (The MathWorks Inc, Natick, MA).

Assuming that the total subharmonic backscattering cross-section is the sum of the subharmonic cross sections of all individual bubbles in the volume of interest at low bubble concentrations [15], a quantifiable parameter termed the fractional blood volumes (FBV) similar to the definition of the fractional moving blood volume [16-18] can be defined. The FBV in an ROI can be estimated as the subharmonic signal intensities from the blood normalized by the intensity of 100 % blood (i.e., a normal blood vessel) obtained at the same depth and with the same overlying tissues [17-18]. Perfusion was estimated from the initial slope of the FBV uptake (rFBV) as:

$$\mathrm{rFBV} = d\mathrm{FBV}/dt \tag{2}$$

where the initial slope was defined as the slope from 10 % to 30 % above baseline (scaling relative to the difference between baseline and peak enhancement). The perfusion estimates were averaged over three injections to obtain the final result.

SHI perfusion data was compared to the gold standard microvascular staining technique using linear regression analysis with p-values less than 0.05 considered significant. The effect of biological variability (dog and location within the kidneys) as well as perfusion state (low versus high perfusion i.e., before and after ligation) on the perfusion estimates was investigated using a two-way analysis of variance (ANOVA; Stata 8.0, Stata Corporation, College Station, TX). The perfusion estimate was the dependent variable, while the individual dog, the measurement location (anterior and posterior) and the perfusion state were considered the independent variables with the two latter being nested within dogs. A mixed effects model was used since locations and perfusion states are fixed and dogs are random. Finally, the perfusion data was split into different locations (anterior and posterior) and different perfusion states (high and low) as well as the four possible combinations of location and perfusion state. Then the linear regression analysis was repeated for each perfusion data subset.

In vivo human experiments

As part of an ongoing study women with breast lesions, who subsequently underwent a breast biopsy, participated in a study of mammography and contrast enhanced breast ultrasound [19]. First, a baseline grayscale scan, which identified the mass or abnormality seen by x-ray mammography, was performed by an experienced sonographer followed by a baseline power Doppler scan. Image parameters such as gain, pulse repetition frequency and filter settings were adjusted for each individual patient to optimize flow visualization and then kept constant. Next, Optison was administered intravenously via a peripheral vein (dose: 0.5 ml), preferably the

antecubital vein. The mass or area of abnormality was videotaped with the gain and power Doppler settings unaltered from the pre contrast settings (except that the gain could be lowered if excessive color blooming occurred due to the contrast agent [20]). This allowed for side-by-side comparison of pre and post contrast power Doppler studies. A second injection was made to acquire grayscale SHI data with the modified Logiq 9 scanner (transmitting/receiving at 4.4/2.2 MHz). Sufficient time (10 – 15 minutes) was allowed between injections to avoid any cumulative effect of contrast and to ensure a return to baseline conditions. All patients subsequently underwent surgical biopsies with a final histopathological assessment, which served as the gold standard.

Digital clips were acquired of each injection and transferred to a PC for off-line analysis. Power Doppler and SHI time intensity curves were determined within each lesion using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Then the perfusion within the breast lesion was estimated using the relationship established in the animal experiments.

5.2 Results and Discussion

In vitro experiments

Figure 2 shows a comparison of the subharmonic backscatter from the contrast agent QFX at insonation powers of 0.4 and 0.6 MPa, respectively. Subharmonic signals from the contrast medium could be observed to increase 22 dB around 1.5 MHz with the increase in pressure. The amplitude of the subharmonic backscatter was about 14 dB lower than the fundamental signal at the 0.6 MPa pressure level (compared to 36 dB below at the 0.4 MPa level). These results were reported at an international conference [10].

Figure 3 shows a comparison of the average subharmonic backscatter from the contrast agent Optison and from distilled water in a single 300 μm tube at an insonation power of 1.5 MPa. Subharmonic signals from the contrast medium could be observed over a range of frequencies around 2 MHz, with the peak signal close to 2 MHz. The amplitude of the subharmonic backscatter was about 10 dB higher than the signal from distilled water at this pressure level. Figure 4 show the subharmonic signal amplitudes (± one standard deviation) detected for insonation pressure amplitudes from 0.3 to 1.5 MPa. For comparison the average backscatter measured from distilled water flowing through the tube is also provided. Only at pressure levels above 0.9 MPa is marked subharmonic signal components measured, with the largest being approximately 10 dB above that of the water.

An important factor, which may affect the subharmonic amplitude, is the velocity of the contrast agent within the tube. The flow velocity in the 300 μm tube was about 12 cm/s. This is much higher than the expected flow velocities in the neovessels feeding tumors. The flow velocity in the dialysis cartridge was estimated at 2 mm/s, by determining the volumetric flow rate through the cartridge and dividing by the total cross-sectional area of all the thin tubes. This velocity is close to the actual flow velocity that one might expect to see in neovessels. Figure 5 shows a comparison of subharmonic backscatter amplitude from distilled water and contrast agent. An increase in the subharmonic backscatter of about 12 dB is observed when the insonation pressure was increased from 0.3 MPa to 1.5 MPa similar to the subharmonic signal components measured in the single 300 μm tube (cf., Fig. 3) [11].

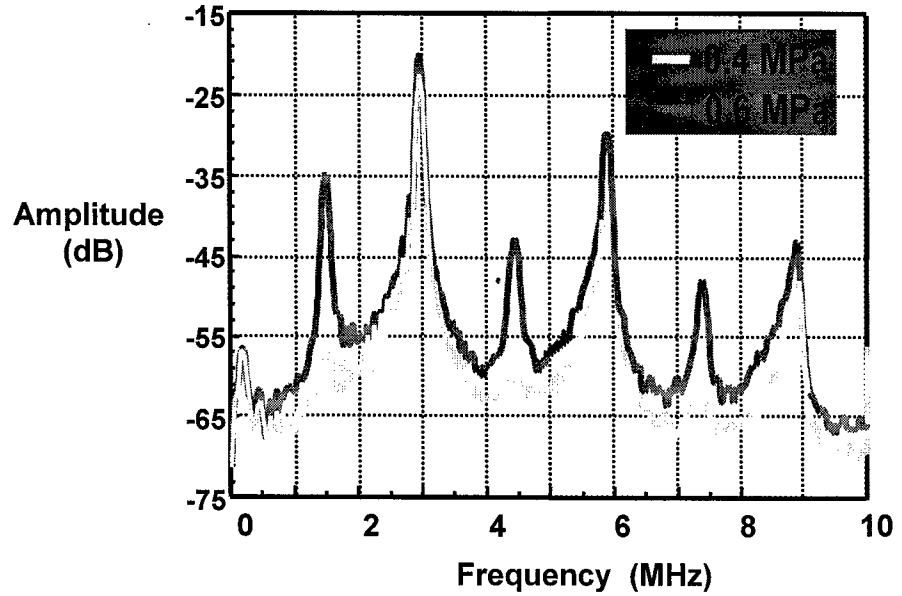

Figure 2. *Signal intensity vs frequency for QFX (concentration 1 μl/ml) obtained at insonation pressures of 0.4 and 0.6 MPa using a 3 MHz pulse of 32 cycles with a 5 Hz PRF.*

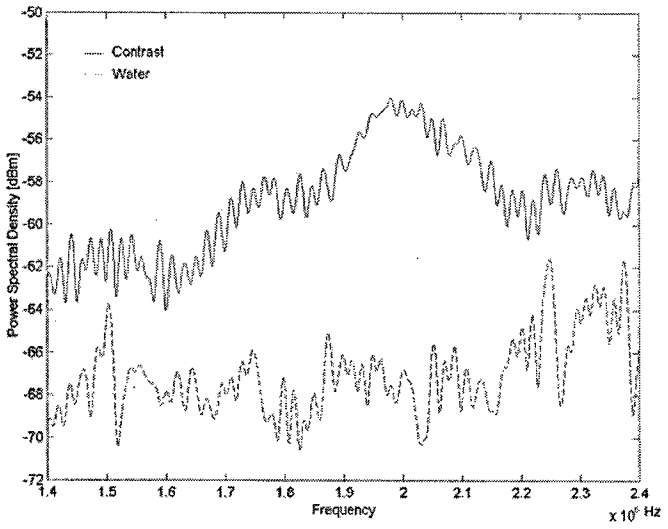

Figure 3. *Power spectral densities averaged over 16 waveforms. The waveforms correspond to backscatter from contrast agent and distilled water, flowing through a tube with internal diameter of 300 μm. The insonation power was 1.5 MPa with a PRF of 50 Hz and insonation frequency of 4 MHz.*

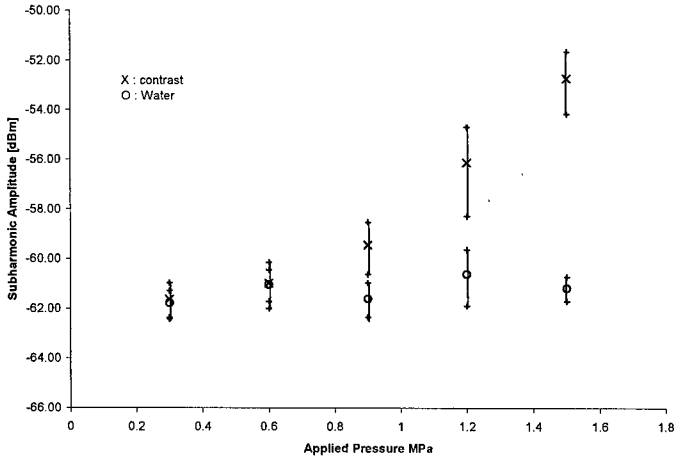

Figure 4. *The average subharmonic backscatter from the contrast agent Optison and from water as a function of the applied pressure. The tube diameter is 300 μm. The vertical bars indicate ± one standard deviation.*

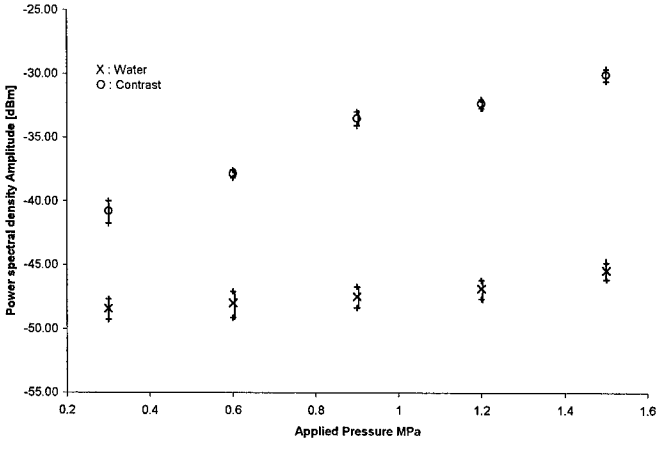

Figure 5. *The average subharmonic backscatter from Optison and from water as a function of the applied pressure in the dialysis cartridge. The internal diameter of the tubes in the cartridge is 200 μm. The vertical bars indicate ± one standard deviation.*

In the flow phantom the contrast kinetics of the microbubbles within the flow was measured for different concentrations of 1% and 2% (by volume) and flow rates of 9.8 and 19.6 ml/min (Figure 6). The subharmonic intensity increased approximately linearly with time as the bubbles flow in and then decay exponentially as bubbles flow out. The slopes of the linear uptake curves were estimated to be 0.0074, 0.016 and 0.015 s^{-1}, respectively, and the decay rates of the wash-out curves were 0.09, 0.09 and 0.17 s^{-1}, respectively, in Figure 6(a-c). The slope is

approximately doubled when either the concentration or the flow rate is doubled. The decay rate depends on flow rate but not on the concentration, as predicted by Schwarz et al [21]. Hence, the decay rate cannot be used as a predictor of local tumor perfusion.

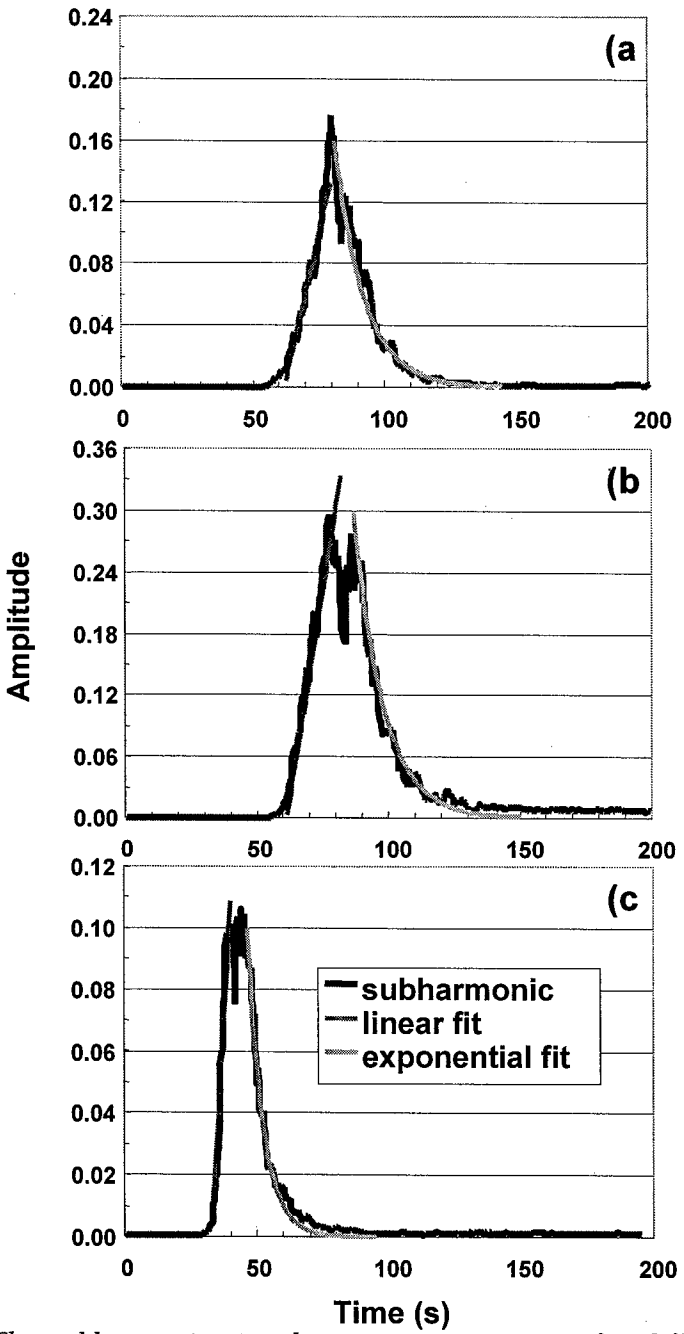

Figure 6. *The subharmonic signal intensity versus time for different concentrations and flow rates: (a) 1% and 9.8 ml/min, (b) 2% and 9.8ml/min, and (c) 1% and 19.6 ml/min.*

In vivo animal experiments

In vivo grayscale SHI following administration of Optison clearly demonstrated flow and, thus, perfusion in the kidney as shown in Figure 7. Notice the fine depiction of small articulate arteries close to the hilum of the kidney (indicating that only minimal, if any, bubble destruction is occurring) and the excellent suppression of tissue echoes. The suppression of tissue signals is even more marked on the baseline image (i.e., prior to administration of Optison), which appears as a completely black image devoid of information (i.e., no subharmonic signals are detectable before the microbubbles arrive). For the sake of brevity the baseline image has, therefore, been omitted.

Following 48 *in vivo* contrast agent injections, a total of 270 SHI time intensity curves were acquired (most of the data from one dog was discarded, due to a problem with the reference blood sample). Additionally, there were 18 curves, which were eliminated due to technical failures (lack of data mainly due to shadowing from the anterior portion of the kidneys and/or very low perfusion states). Hence, after averaging a final 94 perfusion estimates were available for analysis. An example of an original time-intensity curve and the one obtained after smoothing with the fifth-order MA filter is presented in Figure 8. The amplitude variations induced by the respiratory cycle are less pronounced after smoothing.

Overall the SHI perfusion estimates correlated significantly with the gold standard microsphere results ($r=0.57$; $p<0.0001$). The complete perfusion data and the corresponding best linear fit (regression: $Q_p = 0.47 \times rFBV + 1.62$) are depicted in Figure 9. The corresponding root-mean-square-error (RMSE) was 2.48 %. The ANOVA showed the effect of the dogs was significant ($p = 0.006$) and that there were significant interactions between location, perfusion state and dogs ($p < 0.038$). The location and perfusion state on their own were not significant ($p = 0.31$).

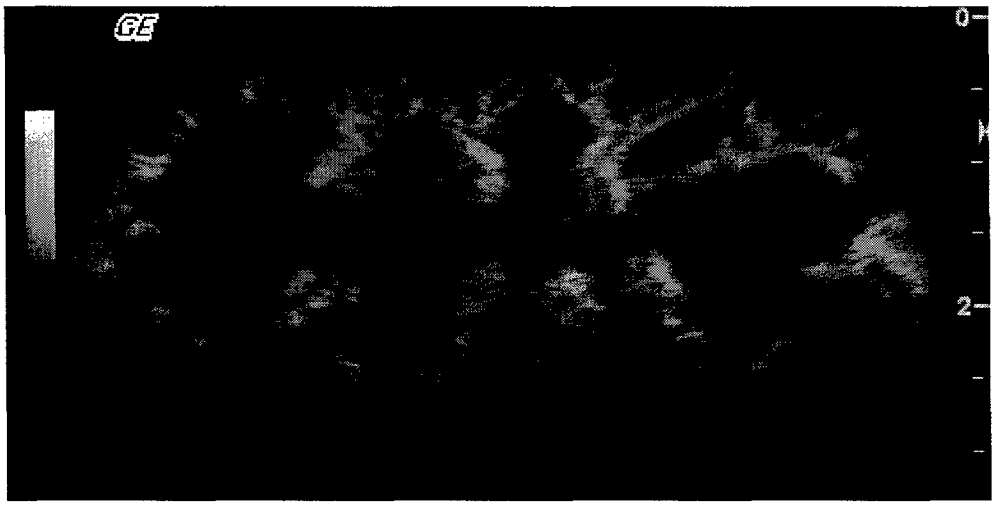

Figure 7. *In vivo SHI post injection of 0.1 ml/kg of Optison showing perfusion in the left kidney obtained with the modified Logiq 9 scanner. The pre injection image is completely black (no subharmonic flow signals are detectable before the microbubbles arrive) and has, therefore, been omitted.*

When the SHI perfusion estimates were split into 8 data subsets and the linear regression analysis repeated for each, significant correlations ($0.47 < r < 0.74$; $p < 0.022$) were found in all cases between the SHI estimates of perfusion and those measured with the gold standard (i.e., the isotope labeled microspheres) as shown in Table 1. The best SHI perfusion estimates occurred for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$; RMSE = 2.39 %); albeit based on a more limited data set ($N = 23$). In other words, when the acoustic shadowing was minimal and the concentration of contrast microbubbles was the highest, SHI perfusion estimates were most accurate.

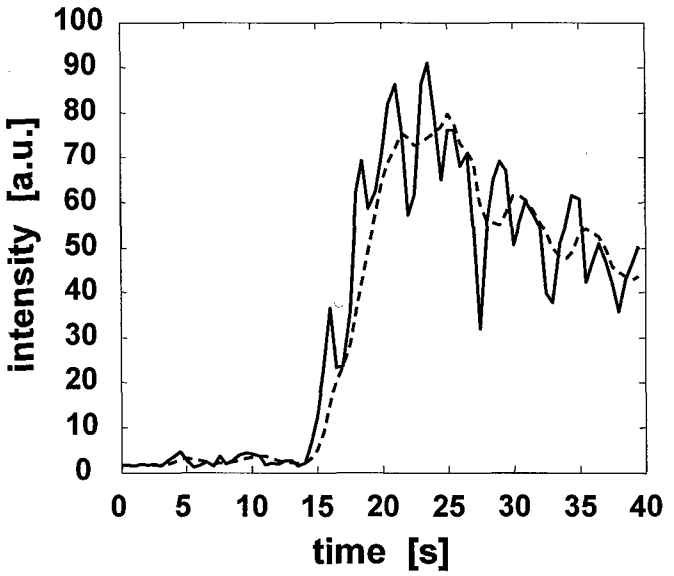

Figure 8. *SHI time-intensity curves before (i.e., original data; solid line) and after smoothing with the fifth-order MA filter (dashed line). The arrival of contrast bubbles approximately 14 s after injection can clearly be seen*

Table 1. Sub-analyses of the *in vivo* SHI perfusion data.

	r value	p value
Anterior	0.56	< 0.0001
Posterior	0.57	< 0.0001
High perfusion	0.61	< 0.0001
Low perfusion	0.64	< 0.0001
Anterior / high	0.73	0.0001
Anterior / low	0.57	0.0037
Posterior / high	0.48	0.0214
Posterior / low	0.71	0.0001

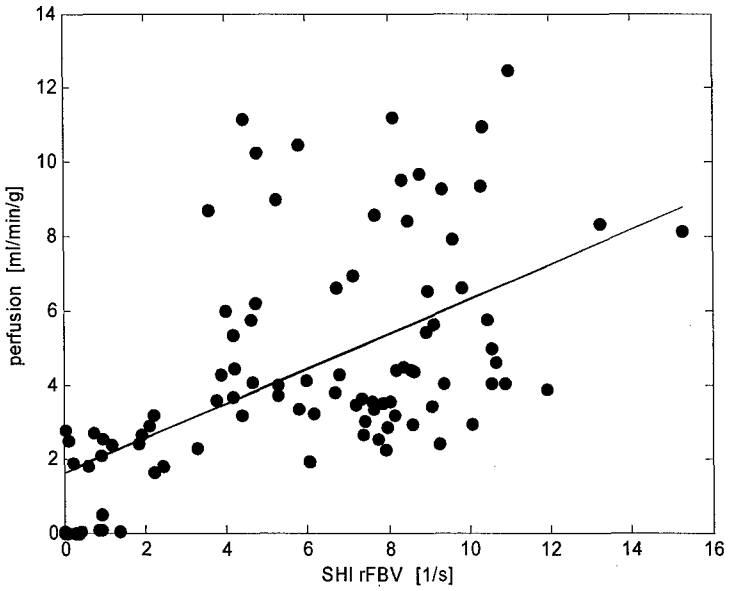

Figure 9. *The microsphere perfusion (in ml/min g) versus all SHI perfusion estimates as well as the best linear fit (solid line).*

There are some limitations to the current study apart from the problems associated with acoustic shadowing and low perfusion states (as mentioned previously). Motion artifacts induced by the dogs breathing were not compensated for when the time-intensity curves were acquired (although the ROI's were chosen large enough to reduce such effects). More importantly, the SHI data were obtained from a single imaging plane and this will induce some additional variability when compared to the microvascular staining technique, which is inherently three-dimensional in nature. To somewhat compensate for this problem, care was taken to both image the largest diameter of the kidneys and to cut the harvested kidneys in that largest diameter (however, some variability is inevitable). The results of the *in vivo* animal experiments have been presented at an international conference and submitted for publication in a peer-reviewed journal [22-23] (see also Appendix II).

Human in vivo experiments

In order to obtain IRB approval for the human component of this project the acoustic power levels of the SHI software on the selected probe (the 7L) was measured. This effort was carried out at GE Medical Systems in Milwaukee during the week of August 11th, 2003. By then all technical and scientific aspects required to enable the initiation of the human clinical trial were completed (as planned and reported in the Annual report of 2003). Subsequently, research agreements between Thomas Jefferson University and GE Medical Systems and Amersham Health, which were required to conduct the human clinical trial at Thomas Jefferson University, took three months to finalize. Following receipt of the fully executed agreements in mid-November, the submission for the Human Subjects Research Review Board (HSRRB) was prepared. The Thomas Jefferson University Institutional Review Board approved protocol, consent form, and case report forms were submitted to the U.S. Army Medical Research and

Material Command on December 22, 2003, but the protocol was not reviewed by HSRRB until March 10th, 2004. Comments from the HSRRB meeting and suggested minor changes were emailed to the Principal Investigator on April 9th. The revisions received final approval by the University's Institutional Review Board in early June, 2004 and was submitted to the HSRRB by June 16th, 2004. The final approval by the HSRRB and, hence, permission to commence the human clinical trial was received by TJU on November 11th, 2004. Following approval by Thomas Jefferson University the recruitment of human subjects commenced by mid-December, 2004.

Given the holiday season this effectively left six (6) months for the human portion of this project. Moreover, the delay in obtaining the necessary administrative approvals also meant that the NIH and DOD funded study into the efficacy of ultrasound contrast in the diagnosis of breast disease that was being conducted at Thomas Jefferson University had ended. Thus, the originally envisaged amalgamation of the NIH/DOD project with this SHI project (including the anticipated cost savings) had to be abandoned. Although the initial grant proposal anticipated a subject population of 50 patients (over 12 months), there were approximately 20 patients screened for enrollment over the 6 months available, but only 6 patients consented and were enrolled.

Six patients with seven benign breast masses were studied (for details see Table 2). In SHI there was an almost complete suppression of tissue signals allowing the lesion vascularity to stand out as demonstrated in Figure 10. This is an important result, since the concentration of contrast agent (4.0 ml of Optison injected into an adult woman) is much lower than in the canine experiments. Moreover, the internal morphology of the vascularity associated with the breast masses was visualized better with SHI than with power Doppler. SHI perfusion estimates obtained in the breast lesions ranged from 1.67 to 2.46 ml/min/g (Table 3). Given that all the studied lesions were benign it is unfortunately not possible to draw any further conclusions regarding the ability of SHI to diagnose breast lesions. However, we intend to continue the human study as an unfunded study for a while longer to enable a sufficient number of subjects (between 12 and 16 patients in total) to be recruited. The human data will then be analyzed, as envisaged in the original grant application, and the results will be submitted for publication at that time.

6. KEY RESEARCH ACCOMPLISHMENTS

- A perfusion phantom with velocities of 2 mm/s (i.e., capillary velocities) was realized based on 200 μm tubes within a dialysis cartridge.
- SHI experiments were conducted with QFX and Optison in the perfusion phantom.
- Up to 12 dB of enhancement was measured for a 1.5 MPa pressure.
- SHI experiments were conducted with Optison and the Logiq 700 scanner in a flow phantom and the contrast uptake slope was found to double with concentration and flow rate.

Table 2. Demographics of the human subjects studied with SHI.

ID	Age [years]	Racial category	Diagnosis
SHI01	37	African-American	Fibroadenoma
SHI02	50	Caucasian	Lymphocytes suggestive of Sinus Histiocytes
SHI03	48	Caucasian	Fibroadipose tissue with benign ductal and lobular elements
SHI04	50	African-American	Two cysts
SHI05	57	Caucasian	Proliferative fibrocystic changes with focal columnar cell hyperplasia, sclerosing adenosis and benign ductal microcalcifications
SHI06	64	Caucasian	Hyalinized fibroadenoma with microcalcifications

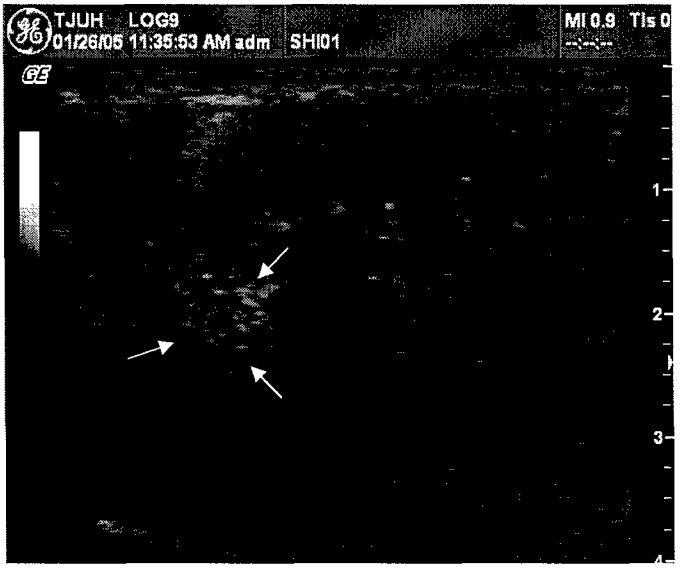

Figure 10. *The first ever case of a breast lesion imaged with SHI in a* ***human****. The fibroadenoma (arrows) is clearly more vascular than the surrounding normal breast tissue.*

Table 3. SHI based estimates of perfusion in the human breast lesions.

Lesion ID	Slope	Q_p [ml/min/g]
SHI01	1.0484	2.11
SHI02	1.7810	2.46
SHI03	1.1746	2.17
SHI04a	0.1069	1.67
SHI04b	0.1196	1.38
SHI05	0.1989	1.71
SHI06	1.6023	2.37

- SHI was performed in 4 dogs and the probe to be used for human studies (the 7L) was selected.
- In vivo, 270 SHI time intensity curves were acquired, which reduced to 94 perfusion estimates after averaging.
- The best SHI perfusion estimates were found for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$); albeit based on a limited data set ($N = 23$).
- Overall SHI perfusion estimates correlated significantly with the results from the neutron activation assay (the gold standard; $r = 0.57$; $p < 0.0001$).
- The first ever *in vivo* human SHI depictions of breast lesion vascularity have been produced.
- Six subjects with 7 benign lesions were studied.
- Perfusion within the lesions was estimated to range from 1.67 to 2.46 ml/min/g.

7. REPORTABLE OUTCOMES

Govind Bhagavatheeshwaran obtained a Master of Science degree in Biomedical Engineering from Drexel University with P.M. Shankar and F. Forsberg (the PI) as his supervisors.

Peer-Reviewed Journal Articles

G Bhagavatheeshwaran, WT Shi, F Forsberg, PM Shankar. Subharmonic generation from contrast agents in simulated neovessels. *Ultrasound Med Biol*, vol. 30, no. 2, pp. 199 – 203, 2004.

F Forsberg, JB Liu, WT Shi, R Ro, KJ Lipcan, X Deng, AL Hall. In vivo perfusion estimation using subharmonic contrast microbubble signals. Submitted to *J Ultrasound Med,* July, 2005.

Abstracts and Proceedings

F Forsberg, G Bhagavatheeshwaran, WT Shi, PM Shankar. Contrast enhanced subharmonic ultrasound imaging. *Proc Era of Hope, DoD Breast Cancer Research Meet,* pp. P29-3, 2002.

F Forsberg, WT Shi. In vivo subharmonic imaging and pressure estimation. *Proc Leading Edge in Diag. Ultrasound*, B. B. Goldberg (ed.), Thomas Jefferson Univ., Philadelphia, pp. I45 - I48, 2003.

WT Shi, Y Liu, Z Lu, F Forsberg, D Zha, JB Liu, BB Goldberg. Nonlinear imaging with a new contrast agent. *Ultrasound Med Biol*, vol. 29, pp. S97, 2003.

F. Forsberg, J. B. Liu, W. T. Shi, R. Ro, K. M. James, X. Deng, A. L. Hall. Subharmonic contrast signals for imaging and quantitative measurements. *Proc Ann Conv Soc Ultrasound Med R.O.C.,* pp. 41-44, 2004.

F Forsberg, JB Liu, WT Shi, R Ro, KM James, X Deng, AL Hall. Perfusion estimation using subharmonic contrast microbubble signals. *Proc IEEE US Symp*, pp. 5 – 8, 2004

F. Forsberg, J.B. Liu, W.T. Shi, R. Ro, K.M. James, X. Deng, A.L. Hall. In vitro and in vivo perfusion estimation using subharmonic ultrasound signals. *J Ultrasound Med,* vol 24, pp. S64, 2005.

F. Forsberg, C. W. Piccoli, R. Ro, K. J. Lipcan, D. A. Merton, J. B. Liu, R. Soparawala, W. T. Shi, A. L. Hall. Contrast Enhanced Subharmonic Breast Imaging: Work in Progress. Accepted for publication in *Proc. IEEE US Symp*, 2005.

Presentations

September 25 – 28, 2002	Era of Hope, Dept. of Defense Breast Cancer Research Meeting, Orlando, FL, USA. • Contrast enhanced subharmonic ultrasound imaging (poster).
May 13 - 16, 2003	The Leading Edge in Diagnostic Ultrasound, Philadelphia, PA, USA. • In vivo subharmonic imaging and pressure estimation.
June 1 - 4, 2003	The 47th Annual Convention of the American Institute of Ultrasound in Medicine, and the 10th Congress of the World Federation for Ultrasound in Medicine and Biology, Montreal, Canada. • Nonlinear imaging with a new contrast agent
September 25, 2003	Breakthrough Seminar 2003, GE Yokogawa Medical Systems, Osaka, Japan. • New Methods and Applications for Ultrasound Contrast Imaging.

September 27, 2003	15th Doppler Ultrasound Meeting, Japanese Ultrasound Society, Tokyo, Japan. • Recent Developments in Contrast Enhanced Ultrasound Imaging – an American Perspective.
October 24, 2003	Biomedical Ultrasound Faculty Group Seminar, Drexel University, Philadelphia, PA, USA. • In Vivo Subharmonic Imaging and Pressure Estimation.
May 11 - 14, 2004	The Leading Edge in Diagnostic Ultrasound, Atlantic City, NJ, USA. • In Vivo Subharmonic Imaging and Perfusion Estimation.
June 28, 2004	Development Therapeutics Program Meeting, Kimmel Cancer Center, Philadelphia, PA, USA. • Ultrasound Contrast Imaging: Towards Functional Imaging and Therapeutics.
August 24 - 27, 2004	IEEE 2004 Ultrasonics Symposium, Montreal, Canada. • Perfusion estimation using subharmonic contrast microbubble signals.
October 15, 2004	Institute of Bioengineering, National Yang-Ming University, Taipei, Taiwan, R.O.C.. • Subharmonic contrast signals for imaging and quantitative measurements.
October 16 - 17, 2004	20th Annual Convention of the Society of Ultrasound in Medicine of the Republic of China, Taipei, Taiwan, R.O.C.. • Subharmonic contrast signals for imaging and quantitative measurements.
October 19, 2004	Dept. of Ultrasound in Medicine, No. 6 People's Hospital, Shanghai, P. R. China. • Subharmonic contrast signals for imaging and quantitative measurements.
March 4 - 5, 2005	10th Ultrasound Contrast Research Symposium in Radiology, San Diego, CA, USA. • Novel Nonlinear Contrast Imaging Modes for Breast lesion Characterization.
May 10 – 13, 2005	The Leading Edge in Diagnostic Ultrasound, Atlantic City, NJ, USA. • Novel Nonlinear Contrast Imaging Modes for Breast Lesion Characterization. • Contrast Enhanced Breast Imaging.

June 19 - 22, 2005 — The 50th Annual Convention of the American Institute of Ultrasound in Medicine, Orlando, FL, USA.

- In vitro and in vivo perfusion estimation using subharmonic ultrasound signals.

8. CONCLUSIONS

A dual-transducer pulse-echo system was built to perform *in vitro* SHI measurements and experiments were conducted using the contrast agent Optison in a perfusion phantom with realistic neovascular flow velocities (around 2 mm/s). Up to 12 dB of subharmonic signal components were measured (Fig. 5). This work was published in an international journal.

The *in vivo* animal experiments have been completed (i.e., task 2 as modified to accommodate a different animal model). The SHI perfusion estimates were in reasonable agreement with a microvascular staining technique ($r = 0.57$; $p < 0.0001$; Fig. 9). Moreover, the best SHI perfusion estimates were found for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$; Table 1); albeit based on a limited data set. This work has been submitted for publication in an international journal.

The acoustic power testing of the SHI software on the 7L probe was completed at GE's testing facility in August 2003. All subsequent efforts were focused on the administrative work required to initiate the clinical trial of SHI in women with breast lesions. The final approval by the HSRRB and, hence, permission to commence the human clinical trial was received by mid-November, 2004. Over a 6 months period six patients with seven benign breast masses were studied. In SHI there was an almost complete suppression of tissue signals allowing the lesion vascularity to stand out (cf., Fig. 10). Moreover, SHI perfusion estimates obtained in the breast lesions ranged from 1.67 to 2.46 ml/min/g (Table 3).

Given that all the studied human breast lesions were benign it is unfortunately not possible to draw any further conclusions regarding the ability of SHI to diagnose breast lesions. However, we intend to continue the human study as an unfunded project to enable a sufficient number of subjects to be recruited. The human data will then be analyzed, as envisaged in the original grant application, and the results will be submitted for publication at that time.

In summary, tasks 1 and 2 have been completed in their entirety along with tasks 3a, 3b, 3c and 3d (albeit for a smaller patient population), while tasks 3e and 3f cannot be completed due to the lack of malignant lesions in the study population (to date).

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Appendix I

The Statement of Work from the original proposal:

Objectives 1 - 2
Task 1: Software development and *in vitro* experiments (months 1 - 24)

a. Develop software for SHI and for FBV estimates to be produced from SHI data (months 1 - 24).
b. Design and implement pulse echo SHI setup (months 1 - 6).
c. Perform *in vitro* flow phantom experiments comparing SHI and FBV estimates to absolute perfusion and flow rates (months 6 - 12).

Objectives 2 - 3
Task 2: Animal experiments and data collection (months 13 - 24)

a. Perform *in vivo* experiments in 12 normal rabbits comparing FBV estimates to absolute flow rates and perfusion obtained with colored microspheres (months 13 - 20).
b. Perform *in vivo* experiments in 6 rabbits with renal VX-2 tumors implanted comparing FBV estimates to absolute tumor perfusion obtained with colored microspheres (months 20 - 24).
c. Evaluate the performance of SHI in the detection of rabbit VX-2 tumors compared to conventional ultrasound imaging, with and without contrast administration, as well as to harmonic imaging (months 13 - 24).

Objectives 4 - 5
Task 3: Human data collection and analysis (months 25 - 36)

a. Recruit 50 - 75 patients, which is about two-thirds of the anticipated number of patients being enrolled in the existing NIH/DOD supported contrast study (months 25 - 36).
b. Perform SHI contrast studies as part of the already funded NIH/DOD project. This involves an extra injection of contrast (within the permitted total dose) and will add no more than 20 minutes to the total duration of the contrast study (months 25 - 36).
c. Research coordinator to collect clinical information, pathology results, etc. (months 25 - 36).
d. Incorporate SHI findings into the existing database developed for the NIH/DOD supported study (months 25 - 36).
e. Perform ROC analysis in collaboration with the statistician (months 30 - 36).
f. Perform remaining statistical analysis in collaboration with the statistician (months 30 - 36).

Appendix II

Copy of the manuscript submitted for peer review and potential publication in Journal of Ultrasound in Medicine:

In Vivo Perfusion Estimation

Using Subharmonic Contrast Microbubble Signals

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Abstract

Objective: To quantify perfusion *in vivo* using contrast enhanced subharmonic imaging (SHI).

Methods: A modified Logiq 9 scanner (GE Healthcare, Milwaukee, WI) operating in grayscale SHI mode was used to measure SHI time intensity curves *in vivo*. Four dogs received intravenous contrast injections (dose: 0.1 ml/kg) and renal SHI was performed. Following 3 contrast injections a microvascular staining technique based on stable (non-radioactive), isotope labeled microspheres (BioPhysics Assay Laboratory Inc, Worcester, MA) was employed to quantify the degree of perfusion in 8 sections of each kidney. Low perfusion states were induced by ligating surgically exposed segmental renal arteries followed by contrast injections and microvascular staining. Digital clips were transferred to a PC and SHI time intensity curves acquired in each section using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). SHI fractional blood volumes (FBVs) were calculated and the perfusion estimated from the initial slope of the FBV uptake averaged over 3 injections. SHI perfusion data was compared to the gold standard using linear regression analysis.

Results: *In vivo* grayscale SHI clearly demonstrated flow and, thus, perfusion in the kidneys with almost complete suppression of tissue signals. In total, 270 SHI time intensity curves were acquired, which reduced to 94 perfusion estimates after averaging. SHI perfusion estimates correlated significantly with microsphere results ($r = 0.57$; $p < 0.0001$). The best SHI perfusion estimates occurred for high perfusions states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$). The corresponding root-mean-square-error was 2.4 %.

Conclusion: SHI perfusion estimates have been obtained *in vivo*. The perfusion estimates were in reasonable to good agreement with a microvascular staining technique.

Keywords: subharmonic imaging, perfusion estimation, ultrasound contrast agent, *in vivo* flow measurements

Short Title: *In Vivo* Subharmonic Perfusion Estimation

Introduction

Nonlinear ultrasound contrast imaging techniques are widely recognized as markedly improving both the sensitivity and specificity of diagnostic ultrasound imaging.[1] At higher acoustic pressures (> 0.5 MPa) microbubble based ultrasound contrast agents act as nonlinear oscillators and produce significant energy components in the received echo signals, spanning the range from subharmonic over fundamental to ultraharmonic frequencies.[1,2] One particular nonlinear contrast imaging technique, which has been extensively studied is second harmonic imaging (HI).[3-6] It is commercially available most commonly in combination with pulse inversion imaging, where a pulse pair 180° out of phase with one another is transmitted and the received signals summed to cancel first (and other odd) harmonics.[7-10]

However, HI (even in combination with pulse inversion imaging) suffers from reduced blood-to-tissue contrast resulting from second harmonic generation and accumulation in tissue. Subharmonic imaging (SHI), transmitting sound pulses at one frequency (f_0) but receiving only echoes at the subharmonic frequency ($f_0/2$), may be an attractive alternative owing to the lack of subharmonic generation in tissue and the significant subharmonic scattering produced by some new contrast agents.[11] Feasibility studies of contrast imaging in SHI mode have been conducted *in vitro* and *in vivo* by our group[11-14] and by others.[15,16]

Quantitative measurements of hydrostatic pressure based on subharmonic signals have been explored in the literature,[17-19] but no one has, to the best of our knowledge, attempted to measure perfusion with SHI. Hence, this project hypothesizes that absolute perfusion (in ml/min/g) can

be quantified *in vivo* using contrast enhanced SHI and investigates the feasibility of perfusion estimation using this novel technique.

Materials and Methods

Four laboratory bred mongrel dogs (mean weight: 21 kg) were used in this project. The dogs were pre-medicated with intramuscular administration of a mixture of 0.04 mg/kg atropine sulfate (Anthony Products, Arcadia, CA), and 0.75 mg/kg acepromazine (Promace; Aveco, Fort Dodge, IA). The dogs were placed on a warming blanket to maintain body temperature within normal range. A facemask with Isoflurane 4 to 5 % (Iso-thesia; Abbott Labs, N.Chicago, IL) was used for induction of anesthesia, which was maintained with 0.5 to 2 % of Isoflurane during the entire procedure. An 18 gauge angiocatheter was placed in a forelimb vein in the dogs for contrast material administration. An arterial catheter was inserted through a carotid as well as through a femoral artery and positioned (under ultrasound guidance) in the left ventricle and the aorta, respectively. The animal studies were performed under supervision of a veterinarian and fully conformed to the National Institutes of Health guidelines for use of laboratory animals. All protocols were approved by the University's Animal Use and Care Committee.

Bilateral renal grayscale pulse inversion SHI was performed using a modified Logiq 9 scanner (GE Healthcare, Milwaukee, WI) with a broad bandwidth linear array (the 7L probe; bandwidth 3 - 7 MHz). The dogs received intravenous injections of the ultrasound contrast agent Optison® (GE Healthcare, Princeton, NJ) at a rate of approximately 1 ml/s and a dosage of 0.1 ml/kg. Optison is approved for use in echocardiography by the U.S. Food and Drug administration (for improved endocardial border delineation). It consists of a sterile, non-pyrogenic suspension of

octafluoropropane filled human serum albumin coated microspheres with a concentration of 6.3 $\times 10^8$ bubbles/ml and the bubbles have mean diameters from 3 to 5 μm with 93 % being smaller than 10 μm.[6] The frequency of insonation was 4.4 MHz and the receive frequency was 2.2 MHz. This corresponds to the resonance frequency of Optison, which is around 2 MHz, and is the frequency range used in our previous SHI work.[11,12] This is also in keeping with the concept that the threshold, above which subharmonic generation occurs, reaches a minimum when the insonation frequency is twice the resonance frequency of the bubbles imaged.[20] This is also an important design criteria for SHI.[11]

The experimental software employed for SHI does not provide any calibrated indication of the acoustic field (such as a mechanical index). Instead, the acoustic output of the 7L transducer was measured in water at 2 cm's depth (corresponding to the average location of a canine kidney from the skin surface) using a 0.5 mm broadband acoustic hydrophone (Precision Acoustics Ltd, Dorchester, UK). The incident acoustic pressure amplitude was 1.48 MPa (peak negative pressure 0.65 MPa), which corresponds to an *in situ* pressure of approximately 0.9 MPa (assuming attenuation to be 0.5 dB/cm MHz). All imaging parameters were kept constant during injections. For each injection the scan plane was selected to correspond to the maximum diameter of the kidney and a 30 to 40 second digital cine-loop clip, covering baseline to beyond peak enhancement, was acquired. Moreover, sufficient time (10 – 15 minutes) was allowed between injections to avoid any cumulative effect of contrast and to ensure a return to baseline conditions.

Following six contrast injections (3 for each kidney), a neutron activation assay technique[21] based on stable (non-radioactive), isotope-labeled microspheres (initially gold microspheres; BioPhysics Assay Laboratory (BioPal) Inc, Worcester, MA) was employed to quantify the perfusion.[22] The microspheres are 15 μm in diameter and are supplied from BioPal suspended in normal saline containing Tween-80 and 0.01 % Thimerosal. Approximately 10 million microspheres (concentration: 2.5 million spheres/ml) were injected into the left ventricle through the myocardial catheter. This is a small number (fewer than the number of red blood cells in 2 ml of blood) and data reported in the literature show that this procedure does not cause clinically significant embolization.[23] Simultaneously, a reference blood sample was drawn from the catheter positioned in the aorta using an automatic withdrawal pump (Syringe Pump 11 Plus; Harvard Apparatus, Holliston, MA). The withdrawal rate was set at 10 ml/min and collected over a one minute interval resulting in a 10 ml reference blood sample.

Next, a surgically exposed segmental renal artery was ligated (with a surgical suture) to reduce perfusion in the kidney (first on the right and then on the left side). On each side three contrast injections as well as transcutaneous SHI were repeated followed by administration of different isotope labeled microspheres (utilizing Lutetium for the right and Samarium for the left side, respectively). As described above 10 million spheres were injected and a reference blood sample acquired for each side. At the completion of the experiments, the dogs were sacrificed using an intravenous injection of Beuthanasia (0.25 mg/kg).

After euthanasia, the kidneys were harvested, cut in half corresponding to the imaging plane and eight small sections (from the upper pole anterior and posterior, mid-upper pole anterior, ...,

lower pole posterior) were extracted. All tissue sections were weighed (the average sample weighed 0.6 g ± 0.17 g) and stored in pre-weighed sample vials. All tissue and blood samples were sent for activation at BioPal's facilities, where they were exposed to a field of neutrons.[21,22] The samples were stored for 48 hours to allow short-lived activation products to decay. After this decay period, spectroscopic analysis was performed on each sample, and corrections were made to account for interradionuclide crossover and tracer decay during the counting period.[22] The results of the assay were reported as the microsphere concentration of each segment normalized to the microsphere concentration measured in the reference blood sample, which provides a measure of absolute perfusion (Qp; in ml/min g) according to:

$$Q_P = \frac{\sum spheres\ in tissue}{\sum spheres\ in reference} \times Q_R \qquad (1)$$
$$weight\ of\ tissue$$

where Q_R is the reference withdrawal rate (in ml/min).

The digital clips obtained from each SHI injection were transferred to a PC for off-line analysis. SHI time intensity curves were acquired in eight regions of interest (ROIs) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) with each ROI corresponding to one of the eight kidney sections (i.e., upper pole anterior, upper pole posterior, mid-upper pole anterior, etc.). To reduce breathing artifacts all time intensity curves were smoothed with a fifth-order moving average filter using Matlab (The MathWorks Inc, Natick, MA).

Assuming that the total subharmonic backscattering cross-section is the sum of the subharmonic cross sections of all individual bubbles in the volume of interest at low bubble concentrations,[24] a

quantifiable parameter termed the fractional blood volumes (FBV) similar to the definition of the fractional moving blood volume[25-27] can be defined. The FBV in an ROI can be estimated as the subharmonic signal intensities from the blood normalized by the intensity of 100 % blood (i.e., a normal blood vessel) obtained at the same depth and with the same overlying tissues.[26,27] Perfusion was estimated from the initial slope of the FBV uptake (rFBV) as:

$$\mathrm{rFBV} = d\mathrm{FBV}/dt \tag{2}$$

where the initial slope was defined as the slope from 10 % to 30 % above baseline (scaling relative to the difference between baseline and peak enhancement). The perfusion estimates were averaged over three injections to obtain the final result.

Statistical Analysis

SHI perfusion data was compared to the gold standard microvascular staining technique using linear regression analysis with p-values less than 0.05 considered significant. The effect of biological variability (dog and location within the kidneys) as well as perfusion state (low versus high perfusion i.e., before and after ligation) on the perfusion estimates was investigated using a two-way analysis of variance (ANOVA; Stata 8.0, Stata Corporation, College Station, TX). The perfusion estimate was the dependent variable, while the individual dog, the measurement location (anterior and posterior) and the perfusion state were considered the independent variables with the two latter being nested within dogs. A mixed effects model was used since locations and perfusion states are fixed and dogs are random. Finally, the perfusion data was split into different locations (anterior and posterior) and different perfusion states (high and low) as well as the four possible combinations of location and perfusion state. Then the linear regression analysis was repeated for each perfusion data subset.

Results

In vivo grayscale SHI following administration of Optison clearly demonstrated flow and, thus, perfusion in the kidney as shown in Figure 1. Notice the fine depiction of small articulate arteries close to the hilum of the kidney (indicating that only minimal, if any, bubble destruction is occurring) and the excellent suppression of tissue echoes. The suppression of tissue signals is even more marked on the baseline image (i.e., prior to administration of Optison), which appears as a completely black image devoid of information (i.e., no subharmonic signals are detectable before the microbubbles arrive). For the sake of brevity the baseline image has, therefore, been omitted.

Following 48 *in vivo* contrast agent injections, a total of 270 SHI time intensity curves were acquired (most of the data from one dog was discarded, due to a problem with the reference blood sample). Additionally, there were 18 curves, which were eliminated due to technical failures (lack of data mainly due to shadowing from the anterior portion of the kidneys and/or very low perfusion states). Hence, after averaging a final 94 perfusion estimates were available for analysis. An example of an original time-intensity curve and the one obtained after smoothing with the fifth-order MA filter is presented in Figure 2. The amplitude variations induced by the respiratory cycle are less pronounced after smoothing.

Overall the SHI perfusion estimates correlated significantly with the gold standard microsphere results ($r = 0.57$; $p < 0.0001$). The complete perfusion data and the corresponding best linear fit (regression line: $Q_p = 0.47 \times rFBV + 1.62$) are depicted in Figure 3. The corresponding root-

mean-square-error (RMSE) was 2.48 %. The ANOVA showed the effect of the dogs was significant ($p = 0.006$) and that there were significant interactions between location, perfusion state and dogs ($p < 0.038$). The location and perfusion state on their own were not significant ($p = 0.31$).

When the SHI perfusion estimates were split into 8 data subsets and the linear regression analysis repeated for each, significant correlations ($0.47 < r < 0.74$; $p < 0.022$) were found in all cases between the SHI estimates of perfusion and those measured with the gold standard (i.e., the isotope labeled microspheres) as shown in Table 1. The best SHI perfusion estimates occurred for high perfusion states in the anterior of the kidneys as shown in Figure 4 ($r = 0.73$; $p = 0.0001$; RMSE = 2.39 %); albeit based on a more limited data set ($N = 23$). In other words, when the acoustic shadowing was minimal and the concentration of contrast microbubbles was the highest, SHI perfusion estimates were most accurate.

Discussion

Perfusion estimation based on the initial slope of SHI time intensity curves has been evaluated *in vivo*. The SHI perfusion estimates were found to be in reasonable agreement with absolute perfusion measurements obtained with the gold standard neutron activation assay technique ($r = 0.57$; $p < 0.0001$; Figure 3). Further analysis of the data split into subsets based on location and perfusion state (and combinations thereof), established that the best SHI perfusion estimates occurred for high perfusion states in the anterior of the kidneys ($r = 0.73$; $p = 0.0001$; Table 1); albeit based on a more limited data set. Not surprisingly, the biological variability caused by the small number of animals studied did have a significant effect on results ($p = 0.006$).

There are some important limitations to the current study apart from the problems associated with acoustic shadowing and low perfusion states (as mentioned previously). Motion artifacts induced by the dogs breathing were not compensated for when the time-intensity curves were acquired (although smoothing was applied and the ROIs were chosen large enough to reduce such effects cf., Figure 2). More importantly, the SHI data were obtained from a single imaging plane and this will induce some additional variability when compared to the neutron activation assay, which is inherently three-dimensional (3D) in nature. To somewhat compensate for this problem, care was taken to image the largest diameter of the kidneys and to cut the harvested kidneys in that largest diameter (however, some unwanted variability is inevitable). In the future, this problem may be eliminated through the use of 3D SHI.

Finally, multiple comparisons may create some additional statistical uncertainty, since the more comparisons the more likely it is that one will be significant by chance. Some statisticians advocate solving this problem with a Bonferroni adjustment, which assigns the traditional 0.05 p-value divided by the number of comparisons (here 8) to be the p-value of significance.[28] However, others argue that such adjustments create more problems than they solve e.g., increased likelihood of finding true differences to be non-significant.[29] We decided to adopt the latter strategy in reporting the results of this paper. However, it should be noted, that even if Bonferroni correction was applied only high perfusion in the posterior of the kidneys would not produce a significant correlation (since $0.05/8 = 0.00625 < 0.0214$ cf., Table 1).

It is known that the decay rate of a time intensity curve cannot be used as a predictor of local perfusion, since the decay rate depends on flow rate but not on the concentration.[30] Hence, this paper proposes perfusion estimation based on the initial slope of SHI time intensity curves. Contrast enhanced perfusion estimation to date has mainly relied on bubble destruction and replenishment techniques.[31-35] While most studies have attempted to quantify blood flow in ml/min rather than perfusion in ml/min/g,[32,33] the group at University of Virginia estimated absolute myocardial perfusion *in vivo* (in canines) using intermittent grayscale HI and power pulse inversion imaging.[34,35] They reported a correlation coefficients of 0.53 to 0.96 compared to radiolabeled microspheres. However, these results were obtained in an open chest model during contrast infusion and were all based on less than 20 data points. Moreover, in the study with the best correlation (an r of 0.96) the flow through the coronary artery being investigated was kept constant by an external flow pump,[34] which may explain why their results are better than the ones reported here for external imaging under pulsatile flow conditions.

In conclusion, we have described a novel method for contrast enhanced perfusion estimation based on the initial slope of time intensity curves obtained from grayscale SHI. The method was tested by obtaining *in vivo* SHI perfusion estimates in canines. The *in vivo* perfusion estimates were in reasonable to good agreement with a neutron activation assay (r from 0.57 to 0.73), indicating that SHI perfusion estimation may become a viable clinical tool in the future.

Acknowledgments

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Table 1. Regression results from the sub-analyses of the SHI perfusion data

	r-values	p-values
Anterior	0.56	< 0.0001
Posterior	0.57	< 0.0001
High perfusion	0.61	< 0.0001
Low perfusion	0.64	< 0.0001
Anterior / high	0.73	0.0001
Anterior / low	0.57	0.0037
Posterior / high	0.48	0.0214
Posterior / low	0.71	0.0001

Captions

Figure 1. *In vivo* grayscale SHI post injection of 0.1 ml/kg of Optison showing perfusion in the left kidney.

Figure 2. SHI time-intensity curves before (i.e., original data; solid line) and after smoothing with the fifth-order MA filter (dashed line) to reduce breathing artifacts. The arrival of the contrast bubbles approximately 14 s after injection can clearly be seen.

Figure 3. The SHI perfusion estimates versus microsphere perfusion (in ml/min g) as well as the best linear fit (solid line). The number of data points is 94.

Figure 4. The SHI perfusion estimates versus microsphere perfusion (in ml/min g) obtained in the anterior of the kidneys at high flow rates as well as the best linear fit (solid line). The number of data points is 23.

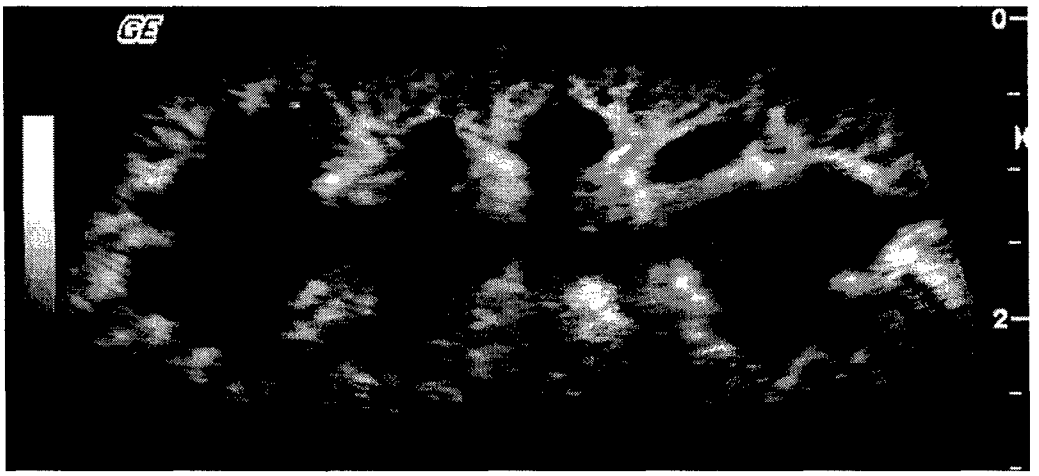
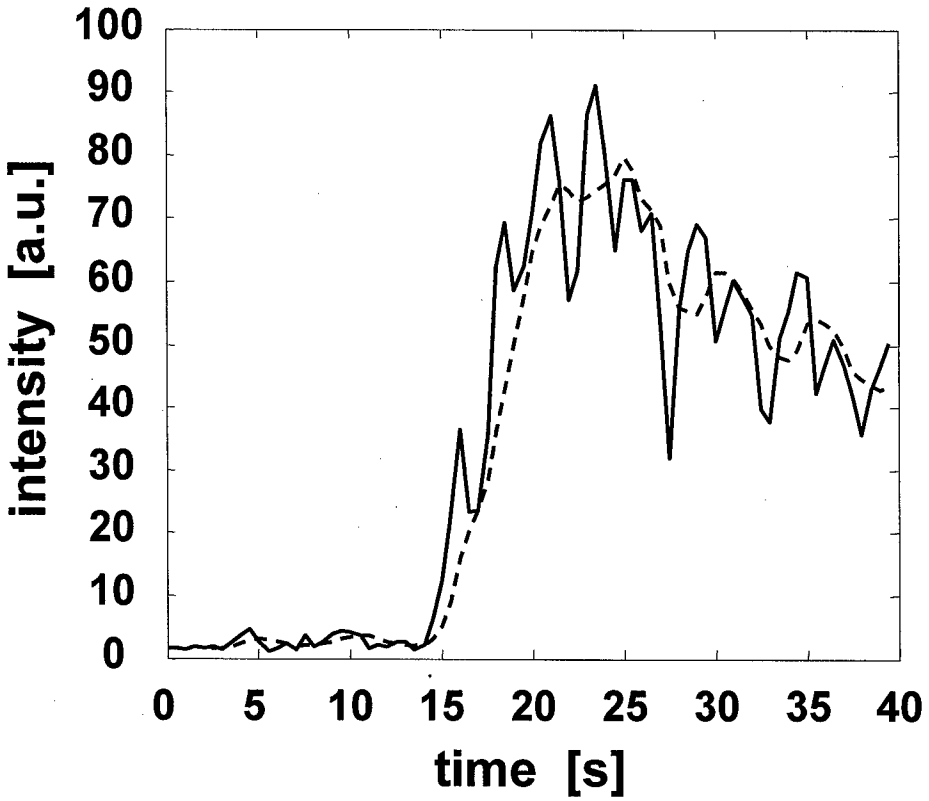


Figure 1. *In vivo* grayscale SHI post injection of 0.1 ml/kg of Optison showing perfusion in the left kidney.



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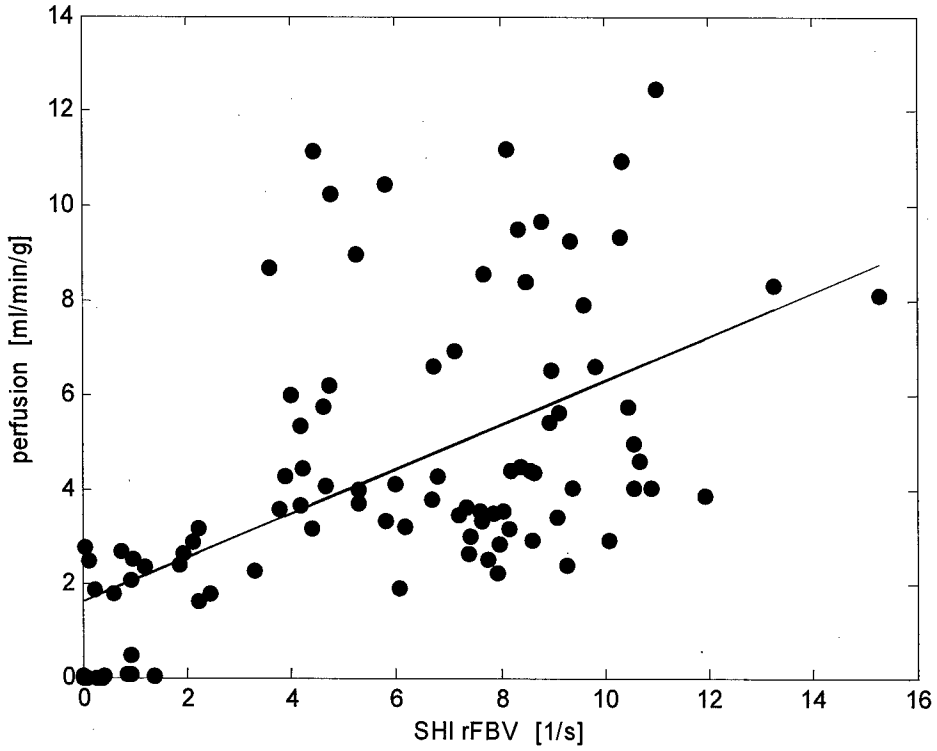

Figure 3. The SHI perfusion estimates versus microsphere perfusion (in ml/min g) as well as the best linear fit (solid line). The number of data points is 94.

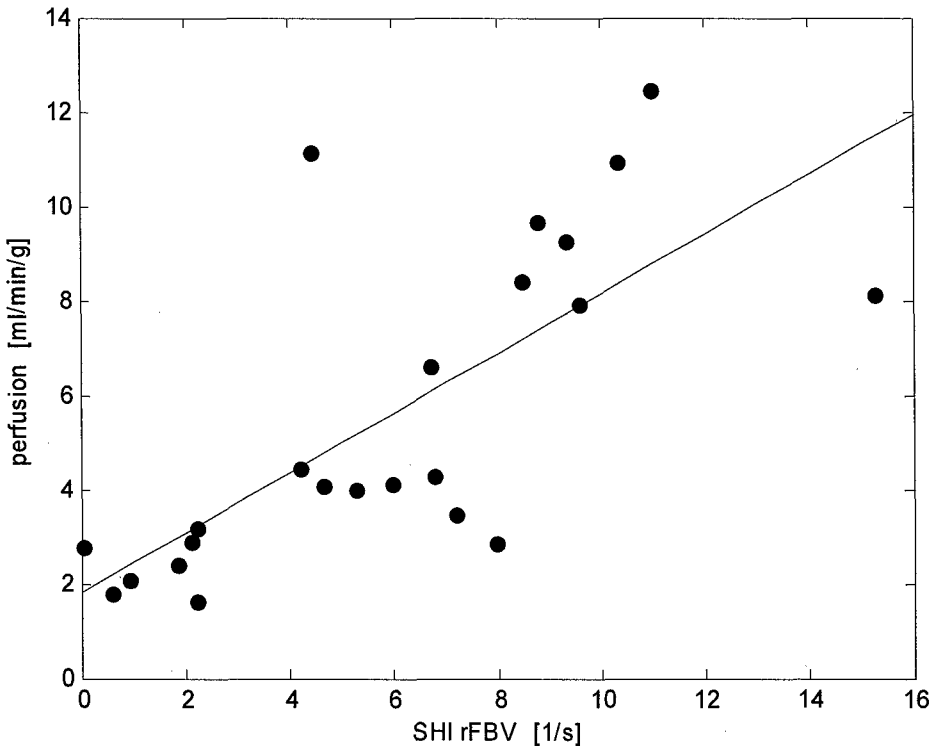

Figure 4. The SHI perfusion estimates versus microsphere perfusion (in ml/min g) obtained in the anterior of the kidneys at high flow rates as well as the best linear fit (solid line). The number of data points is 23.